Differential Activation of Migration by Hypoxia in Keratinocytes Isolated from Donors of Increasing Age: Implication for Chronic Wounds in the Elderly

Yu-Ping Xia,[1] Yanan Zhao, J.W. Tyrone, Alex Chen and Thomas A. Mustoe

Division of Plastic Surgery and Reconstructive Surgery, Department of Surgery, School of Medicine, North-western University, Chicago, Illinois, U.S.A.

Chronic wound healing conditions are often observed in elderly patients with poor tissue oxygenation. Impaired re-epithelialization is a hallmark of these wounds, which is seen in both clinical studies and in our animal models of impaired healing. To investigate the pathogenic mechanism of chronic wounds, we studied the effect of hypoxia on migration of keratinocytes isolated from human donors of increasing age. Keratinocytes from elderly donors had depressed migratory activity when exposed to hypoxia, as opposed to an increase in migration in young cells. Analysis of underlying biochemical changes demonstrated a differential activation of matrix metalloproteinases by hypoxia in keratinocytes isolated from the young and the old. Matrix metalloproteinases-1 and –9 and tissue inhibitor of matrix metalloproteinase-1 were strongly upregulated by hypoxia in young cells, whereas no induction was observed in aged cells. Furthermore, transforming growth factor-β1 signaling appears to be involved in the keratinocyte differential response to hypoxia, as transforming growth factor-β type I receptor was upregulated by hypoxia in young cells, while there was no induction in aged cells. Transforming growth factor-β neutralizing reagents blocked hypoxia-induced matrix metalloproteinase-1, matrix metalloproteinase-9 expression, and hypoxia-induced cell migration as well. Our results suggest that an age-related decrease in response to hypoxia plays a crucial part in the pathogenesis of retarded re-epithelialization in wound. ***Key words: keratinocyte migration/matrix metalloproteinase/TGF-β. J Invest Dermatol 116:50–56, 2001***

Clinical observations suggest that the development of chronic wounds frequently associates with persistent low tissue oxygen supply (hypoxia). The prolonged tissue hypoxia exposes wounds to bacterial infection, a prolonged inflammatory response, and eventually tissue necrosis (Niinikoski *et al*, 1972; Franklin and Poyton, 1996). The elderly population accounts for a large portion of this morbidity (Frantz and Gardner, 1994; Van de Kerkhof *et al*, 1994). Despite the debilitating effect of chronic wounds in the elderly, the pathogenesis of chronic wounds is poorly understood.

Consistent with clinical observations, compelling evidence from laboratory studies have shown that age affects wound healing in several aspects: (i) sprouting of aged microvessels was significantly less than the sprouting of young microvessels (Arthur *et al*, 1998); (ii) increased gelatinase and collagenase levels in skin of aged donors (Ashcroft *et al*, 1997a) and in wound fluid from chronic leg ulcers (Wysocki *et al*, 1993; Weckroth *et al*, 1996); (iii) decreased TIMP (tissue inhibitor of matrix metalloproteinase) levels in the skin of aged donors (Ashcroft *et al*, 1997b); and (iv) reduced deposition of matrix components and re-epithelialization (Ashcroft *et al*, 1997c). Our study presents novel observations with respect to age contribution to the altered migration in response to hypoxia. This age-modulated hypoxia response causes imbalance of matrix metalloproteinase (MMP) and TIMP expression. Our data also indicate the transforming growth factor (TGF) -β signaling pathway as a potential mediator of downstream MMP/TIMP imbalance that ultimately impairs re-epithelialization.

To investigate pathologic mechanism of chronic wounds, we studied the migration of skin keratinocytes and associated biochemical changes in a hypoxic *in vitro* wound healing environment. During normal process of wound healing, basal keratinocytes begin to migrate over the provisional wound bed within several hours after injury. Over the next few days, in the absence of complications, the migrating keratinocytes proliferate to re-establish the epithelium along with the basement membrane (Grinnell, 1992; Cavani *et al*, 1993). Studies have shown that migration of keratinocytes depends on the function of MMP (Pilcher *et al*, 1997). MMP are a family of zinc-dependent endopeptidases that have previously been implicated in the pathobiology of chronic wounds, because a high level of MMP has been detected in exudate and wound tissue (Wysocki *et al*, 1993; Moses *et al*, 1996; Vaalamo *et al*, 1996; Weckroth *et al*, 1996; Ashcroft *et al*, 1997a). These proteinases are needed for extracellular matrix cleavage during re-epithelialization (Pilcher *et al*, 1997), as

Manuscript received July 29, 1999; revised October 13, 2000; accepted for publication October 17, 2000.

Reprint requests to: Dr. T. Mustoe, Division of Plastic Surgery and Reconstructive Surgery, Department of Surgery, School of Medicine, North-western University, 675 N St Clair 10-250, Chicago, IL 60611-3042, U.S.A.

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase.

[1]Present address: Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Rd, Tarrytown, NY 10567, U.S.A.

well as the remodeling phase of wound healing (Agren, 1994). Several forms of MMP have been implicated to play important parts in wound repair. Interstitial collagenase MMP-1, a protease that cleaves fibrillar collagens types I, II, and III at a specific locus in their triple helical domains (Welgus *et al*, 1981; Wu *et al*, 1990; Liu *et al*, 1995), is expressed by migrating basal keratinocytes in all types of wounds with a breached basement membrane (Vaalamo *et al*, 1997; Sudbeck *et al*, 1997). Mutations that block the proteolytic activity of MMP-1 inhibit the migration process of keratinocytes on a provisional wound surface (Pilcher *et al*, 1997; Sudbeck *et al*, 1997). Other studies have shown that wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases, MMP-2 (72 kDa) and MMP-9 (92 kDa) (Wysocki *et al*, 1993; Moses *et al*, 1996; Weckroth *et al*, 1996; Ashcroft *et al*, 1997c). MMP-2 and MMP-9, also known as gelatinase A and B, respectively, cleave type I, type IV and V collagens, and elastin (Salo *et al*, 1994). MMP-2 was recently described specifically to cleave laminin-5 (Giannelli *et al*, 1997), a major epidermal basement membrane component in intact skin (Carter *et al*, 1991).

In the extracellular environment, the activation of metallo-enzymes is regulated, in part, by TIMP. TIMP-1 is a 29 kDa N-glycosylated protein that forms high-affinity, noncovalent complexes with pro-MMP-9 and active MMP-1, -3, and -9, and inhibits the catalytic activity of these MMP (Woessner, 1991; Murphy and Reynolds, 1993). Another physiologic inhibitor of MMP, TIMP-2, is a 22 kDa protein that forms complexes with pro-MMP-2 and active MMP-2 (Murphy and Reynolds, 1993; Howard and Banda, 1991). Studies have shown that TIMP-1 expression was not detected in chronic wound biopsies (Vaalamo *et al*, 1996). In acute wounds, however, TIMP-1 was expressed at the epithelial edges, colocalizing with MMP-1 and MMP-9 (Vaalamo *et al*, 1996; Madlener *et al*, 1998). Thus, the level of TIMP may have a profound effect on re-epithelialization of wounds.

The potential involvement of TGF-β signaling in the hypoxic response was also tested. This is based on our previous study using an animal model of ischemia-impaired wound healing (Wu *et al*, 1999). We have documented (in rabbit wound healing) that the level of TGF-β1 mRNA increased significantly in ischemic over the nonischemic wounds of young animals. In contrast, there was no obvious TGF-β induction in ischemic wounds of aged animals (Wu *et al*, 1999). Other studies have shown that TGF-β1 regulates MMP mRNA expression in a cell-type-specific manner. In keratinocytes, TGF-β1 induces the expression of MMP-1 and MMP-9 (Mauviel *et al*, 1996; Uria *et al*, 1998).

Despite the debilitating effect of chronic wounds in elderly patients, the pathophysiologic factors that cause the nonhealing conditions are unclear. In this study, we examined the effect of age and hypoxia on keratinocyte migration. A reduced migration was observed under hypoxia in cultured keratinocytes isolated from aged donors. This finding was consistent with a previous observation of impaired epithelialization in an animal model of ischemic wounds (Wu *et al*, 1999). Here we examined the migratory response of aged keratinocytes to hypoxia, and a potential regulatory mechanism through the action of MMP and TIMP. We also found that TGF-β neutralizing reagents repressed hypoxia-induced MMP-1 and –9 expression and hypoxia-modulated keratinocyte migration.

MATERIALS AND METHODS

Culture of keratinocytes Primary human skin keratinocyte cultures were established from skin biopsy samples of healthy donors undergoing elective surgical procedures. All tissue was collected in accordance with the guidelines of the North-western University Human Subjects Review Committee. Skin tissue was first digested with dispase to remove the dermis. The remaining epidermis was subsequently digested with trypsin to release keratinocytes from tissue. To maintain cell phenotype in culture, low passage cells (passage 2) were used in our experiments. Cultures were maintained in keratinocyte-SFM medium (Gibco BRL, Gaithersburg, MD) and the medium was changed every other day. Age groups were classified as young (20–39 y old), middle (40–59 y old), and old age (≥ 60 y old). A total of seven different donors were used for each age group, and the results were uniformly consistent, although the magnitudes varied. The aged cells retained their "aged" phenotype *in vitro*. They grew more slowly, quickly became senescent (after three to four passages), and adhered more slowly.

Hypoxic cell culture To subject cell cultures to hypoxia, keratinocytes were placed in a large hypoxia incubator (Coy Laboratory Products, Glass Lake, MI), which allows precise oxygen and temperature regulation whereas permitting media change and other manipulations through a gloved box. The incubator was pre-equilibrated with a gas mixture of 5% CO_2/95% N_2 to achieve an O_2 level of 1%. Culture medium was flushed with N_2, then pre-equilibrated under 1% oxygen tension for 48 h prior to use and the hypoxia incubator is monitored and maintained at this oxygen level throughout the experiment.

Gold salt migration assay Keratinocyte migration was assessed using the method of Albrecht-Buehler as modified by Woodley (Woodley *et al*, 1988). Briefly, colloidal gold salts were immobilized on coverslips and covered with extracellular matrix protein type I collagen (15 μg per ml). Early passage keratinocytes were plated on to the coverslips and allowed to migrate for 20 h. The cells were fixed in 0.1% formaldehyde in phosphate-buffered saline and examined under dark field optics with a video camera attached to a computer equipped with a frame grabber. The computer analyzed 15 nonoverlapping fields in each experimental condition with NIH Image 1.4 and determined the area of each field consumed by cell migration tracks as a percentage of the entire field, termed the migration index. The lengths of individual tracks were also calculated. All migration assays were performed at 1% oxygen tension for hypoxia and 21% oxygen tension for normoxia.

Antibodies and reagents Antibodies against MMP-1, -2, and -9 and TIMP-1 and -2 (Oncogene Research, Cambridge, MA) were used at 1:100 dilution in immunoblotting. Peroxidase-conjugated rabbit antimouse IgG (DAKO, Carpinteria, CA) was used at 1:5000 dilution as a secondary antibody. TGF-β RI and II antibodies (Santa Cruz, Santa Cruz, CA) were used at 1:200 dilution for immunoblotting. Recombinant human TGF-β sRII/Fc chimera is commercially available from R&D Systems (Minneapolis, MN). Neutralizing TGF-β antibody is a generous gift from Genzyme (Cambridge, MA).

Immunoblotting analysis Expression levels of keratinocyte MMP and TIMP were quantitated by immunoblotting analysis. For the detection of MMP, aliquots of equal amounts of protein extracts were resolved by 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes in 20 mM Tris–HCl, pH 8.0, 150 mM glycine, 20% (vol/vol) methanol. The membranes were blocked with 5% (vol/vol) nonfat dry milk in Tris-buffered saline (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20), incubated with MMP-1, -9, or -2 specific antibody (diluted 1:100), washed, incubated with horseradish peroxidase anti-immunoglobulin conjugate (diluted 1:5000) and extensively washed. Protein bands were visualized using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). For detection of TIMP, NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, CA) was used for electrophoresis and protein was transferred in NuPAGE transfer buffer (Novex) and immunoblotted under the same conditions as described above.

Zymography of MMP activity Human keratinocytes were plated on type I collagen at a density of 17,500 cells per cm^2. The cells were incubated under normoxic and hypoxic conditions for 20 h before collecting conditioned culture medium. Gelatinolytic proteinases were assayed by gelatin-substrate enzymography. Gelatin was melted at 50°C for 20 min and added at a 1.5% final concentration to a 10% acrylamide gel. The conditioned media was concentrated using a Centricon-10 microconcentrator (Millipore, Bedford, MA). Aliquots of the media were prepared for electrophoresis without heating or reducing reagents. After sodium dodecyl sulfate–polyacrylamide gel electrophoresis, sodium dodecyl sulfate was removed from the gels by 2.5% (vol/vol) Triton X-100 washes (2 × 20 min), the gels were incubated in assay buffer (15 mM Tris–HCl, pH 7.4, 5 mM $CaCl_2$) at 37°C for 24 h. The reaction was stopped by staining the gels with Coomassie Brilliant Blue (BioRad, Hercules, CA). Gelatinolytic activity was detected as clear bands against the Aqua Blue-stained gelatin background.

Densitometry To quantitate the intensity of individual bands on autoradiographs, the optical density was measured by scanning the films with a Bio-Rad BLS-670 densitometer (Bio-Rad) and analyzed using the computer software program Molecular Analyst (BioRad).

Statistical analysis All data presented as mean ± SEM. Differences between means of two age groups were evaluated by a paired two-tailed Student's t test, with the aid of Excel version 5.0 (Microsoft, Redmond, WA). $p \leqslant 0.05$ was considered statistically significant. Each test using cultured keratinocytes was repeated at least three times to include cells isolated from different donors.

RESULTS

Migration of keratinocytes in response to hypoxia One of the crucial events in re-establishing the basement membrane after cutaneous injury is the migration of basal keratinocytes over the dermal matrices. To assess if the migration of aged keratinocytes is altered by hypoxia, we studied cell migration under hypoxia and normoxia using a primary culture of keratinocytes. Human keratinocytes isolated from healthy aged donors were plated on a type I collagen-coated surface and cultured under either hypoxia (1% oxygen) or normoxia (21% oxygen) for 20 h. The migration index was determined by gold salt migration assay (O'Toole, 1997), which allows the measurement of individual phagokinetic tracks of a single cell. Interestingly, the aged cells showed a significant reduced migration when exposed to hypoxia (**Fig 1*A*, *B***), whereas the control young keratinocytes showed an upregulation of migration (**Fig 1*C*, *D***) as previously reported (O'Toole, 1997). The reduced migration was not due to a general cytotoxic effect of hypoxia exerted on aged cells, as the aged cells seemed to have a normal degree of viability under hypoxia as assessed by Trypan Blue staining (data not shown).

Modulation of MMP expression by hypoxia of keratinocytes cultured on a type I collagen surface As MMP-1 activity is required for keratinocyte migration on type I collagen matrix (Pilcher *et al*, 1997), we first investigated the MMP-1 expression in response to hypoxia in several different age groups. Keratinocytes from donors of increasing age were plated on a type I collagen surface, allowed sufficient time to attach, and transferred to either a hypoxic or normoxic condition. Conditioned culture supernatants were collected 20 h after exposure to hypoxia or normoxia and analyzed for MMP-1 expression by immunoblotting. Expression of both the active form (42–46 kDa) and inactive form (52–57 kDa) of MMP-1 increased with hypoxia in young cells, but not in aged cells under the same low oxygen tension (**Fig 2**). These results suggest that MMP-1 is likely to play an important part in hypoxia-modulated keratinocyte migration on collagen matrix.

We also examined the hypoxia-modulated expression of MMP-2 and MMP-9, gelatinases that become activated in wound repair. Gelatin zymography was carried out to determine the protein level of active and inactive forms of MMP. The protocol involves in-gel activation of MMP by incubating in assay buffer as specified in *Materials and Methods* section. We found that the expression of the active form of MMP-9 (83 kDa) is predominant over the inactive form (92 kDa) on zymography (**Fig 3*A***). Zymography demonstrated that the active form of MMP-9 was stimulated by hypoxia in young cells cultured on type I collagen. On the contrary, hypoxia repressed the expression of MMP-9 in aged keratinocytes (**Fig 3*A***). We also noticed that there was a higher level of MMP-9 in aged keratinocytes compared with the young cells under normoxia (**Fig 3*A***, *lanes 1* and *5*). The inactive form of MMP-9 (92 kDa) was examined by immunoblot using an antibody that specifically recognizes this form of the enzyme. The inactive form of MMP-9 showed a similar pattern of regulation by hypoxia/normoxia as the active form (**Fig 3*B***). There was little if any difference detected for MMP-2 in response to hypoxia (**Fig 4**); however, aged cells displayed an overall higher level of MMP-2 expression compared to young cells (**Fig 4**, *lanes 1*, *2 vs 5*, *6*). **Table I** showed the mean value of the MMP expression ratio under hypoxia *vs* normoxia.

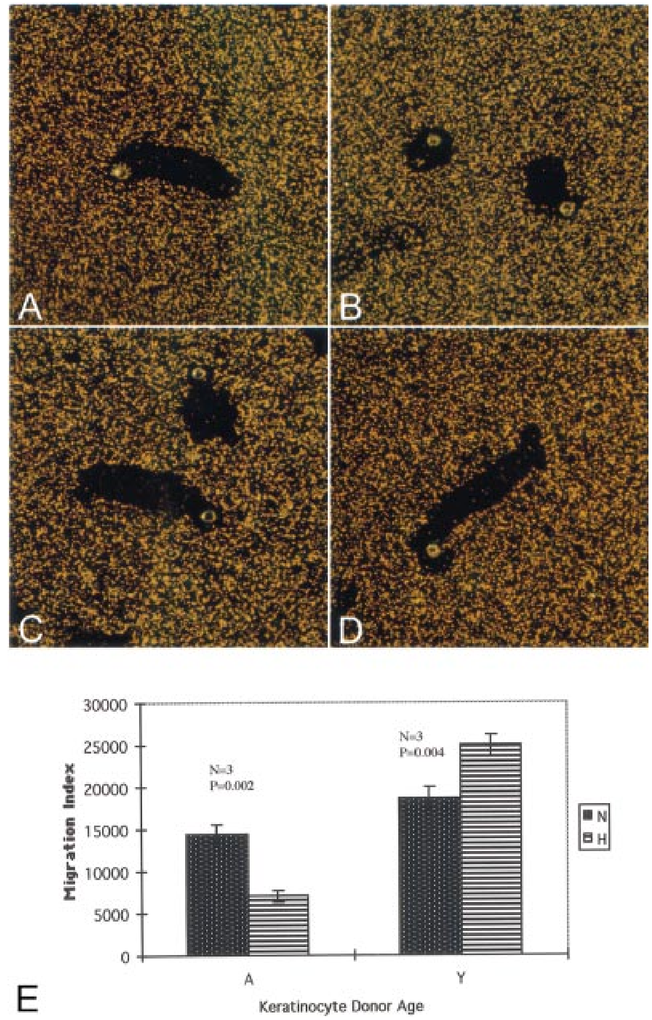

Figure 1. Differential effect of hypoxia on migration of keratinocytes isolated from donors of young and old age. A representative photograph of migration tracks are shown by gold salt migration assay. Human keratinocytes isolated from aged donors (more than 60 y old) were plated on type I collagen surface and allowed to migrate under both normoxic and hypoxic conditions (*A* and *B*). Migration of keratinocytes from young donors (20–39 y old) was analyzed in parallel as control (*C* and *D*). Cells were cultured under normoxia (21% oxygen, *A* and *C*), or hypoxia (1% oxygen, *B* and *D*) for 20 h before fixed in 0.1% formaldehyde and examined under dark field optics for cell migration. The bar graph shows the quantitative results of migration of the young (Y) and the aged (A) keratinocytes. Values are mean ± SEM of over three independent experiments.

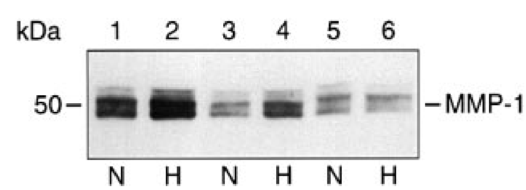

Figure 2. Differential induction of MMP-1 expression by hypoxia in keratinocytes of young and old age. Donor keratinocytes from young (*lanes 1* and *2*), middle age (*lanes 3* and *4*), and age donors (*lanes 5* and *6*) were plated on type I collagen surface in duplicate, and transferred to hypoxic (H) or normoxic (N) culture incubator after cell attachment. Conditioned culture supernatants were collected 20 h after exposure to different oxygen tension and analyzed for MMP-1 expression by immunoblotting. A representative experiment using donor from each age group is shown.

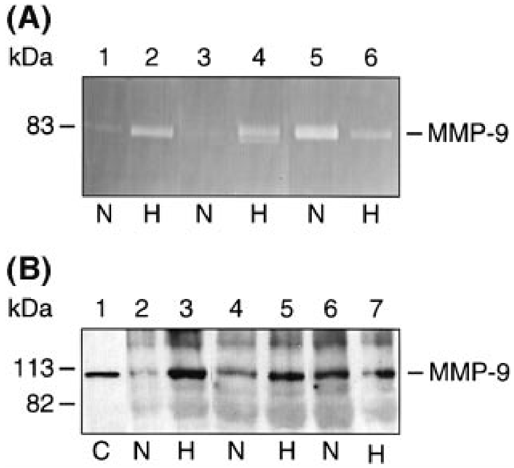

Figure 3. Differential induction of MMP-9 expression by hypoxia in keratinocytes of young and old age. (*A*) Gelatinolytic activity of conditioned culture supernatant of keratinocyte under hypoxia. Conditioned culture supernatants of keratinocytes from donors of increasing age: young (*lanes 1* and *2*), middle age (*lanes 3* and *4*), and old age (*lanes 5* and *6*) were collected under hypoxia (H, 1% oxygen) or normoxia (N, 21% oxygen) 20 h after exposure to different oxygen tension. (*B*) Immunoblot of keratinocyte-conditioned culture supernatant with anti-MMP-9 antibody. The same conditioned culture supernatants (young, *lanes 2* and *3*; middle age, *lanes 4* and *5*; old age, *lanes 6* and *7*) were subject to immunoblotting with MMP-9 specific antibody. Conditioned culture medium from HT1080 cells treated with phorbol myristate acetate (100 nM) were used as a positive control for antibody activity (*C*) against MMP-9. All samples were electrophoresed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with anti-MMP-9 antibody. A representative experiment using donor from each age group is shown.

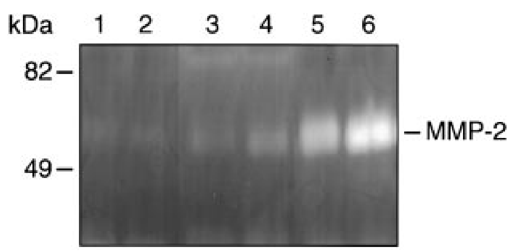

Figure 4. Hypoxia has no significant effect on MMP-2 expression of keratinocytes grown on type I collagen surface. Human keratinocytes were cultured on type I collagen surface and exposed to hypoxia (H) or normoxia (N) for 20 h. Conditioned culture medium was harvested and analyzed for the presence of MMP-2 by gelatin zymogram. Donor age of keratinocytes young (*lanes 1* and *2*), middle age (*lanes 3* and *4*) and old (*lanes 5* and *6*). A representative experiment using donor from each age group is shown.

Differential regulation of TIMP-1 and TIMP-2 expression by hypoxia in keratinocytes from donors of increasing age We also examined the effect of hypoxia on the expression of TIMP-1 and TIMP-2, the physiologic inhibitors of MMP. Interestingly, under hypoxia and on a type I collagen surface, we found that young keratinocytes expressed increased levels of TIMP-1, a physiologic inhibitor that colocalizes and forms inactive complexes with MMP-1 and MMP-9 at the migrating front of the epithelium (**Fig 5*A***). There was a moderate induction in keratinocytes from the middle-aged donors and no induction in the aged keratinocytes (**Fig 5*A***). TIMP-2, which forms a complex with MMP-2, showed varying degrees of decreased expression under hypoxia among different groups (**Fig 5*B***).

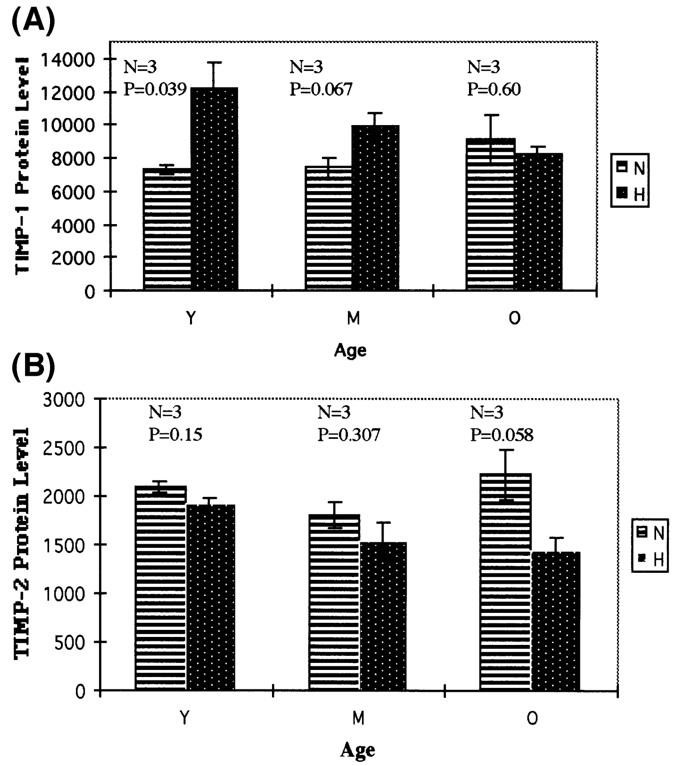

Figure 5. Hypoxia-modulation of TIMP-1 and TIMP-2 expression in keratinocytes isolated from donors of increasing age. (*A*) Donor keratinocytes of young (Y), middle age (M), and old age (O) were plated on type I collagen surface, allowed sufficient time to attach, and transferred to hypoxic (H) or normoxic (N) culture incubator. Conditioned culture supernatants were collected 20 h after exposure to different oxygen tension and analyzed for TIMP-1 expression by immunoblotting. Values are mean ± SEM of three independent experiments. (*B*) Hypoxia-modulated TIMP-2 expression of keratinocytes isolated from donors of increasing age. Donor keratinocytes of young (Y), middle age (M), and old age (O) were plated on type I collagen surface, allowed sufficient time to attach, and transferred to hypoxic (H) or normoxic (N) culture incubator. Conditioned culture supernatants were collected 20 h after exposure to different oxygen tension and analyzed for TIMP-2 expression by immunoblotting. Values are mean ± SEM of three independent experiments.

Differential induction of TGF-β receptor expression by hypoxia in keratinocytes isolated from young and aged donors Our previous studies using an ischemia-impaired animal model showed that the mRNA level of TGF-β1 increased in ischemic wounds of young animals, whereas no induction occurred in aged animals (Wu *et al*, 1999). To test the possible role of TGF-β signaling components in mediating hypoxia-induced biologic responses, we examined the levels of TGF-β type I and type II receptor on keratinocytes cultured under normoxia and hypoxia (**Table II**). We found a differential induction of TGF-β type I receptor by hypoxia in keratinocytes from young and aged donors. Hypoxia upregulated type I receptor expression in the young cells, whereas there was no significant induction in the aged cells. TGF-β type II receptor, on the other hand, did not show significant upregulation under the same condition in any age group (**Table II**).

Role of TGF-β in hypoxia-modulated MMP expression TGF-β displays a stimulatory effect on MMP-1 (Mauviel *et al*, 1996) and MMP-9 expression in keratinocytes (Salo *et al*, 1991; Rougier *et al*, 1997). To investigate whether TGF-β growth factor–receptor interaction plays a part in hypoxia-induced MMP-1 and -9 expression, we included a soluble neutralizing polypeptide of TGF-β type II receptor (the primary binding receptor of TGF-β) in keratinocyte culture and tested MMP expression under both hypoxic and normoxic conditions. This polypeptide contains

Table I. Hypoxia-induced MMP expression in keratinocytes isolated from donors of increasing age[a]

H/N (Fold)	MMP-1	MMP-9	MMP-2
Young	4.43±0.583	5.74±0.422	1.10 ±0.113
Middle Age	2.33±0.263	2.11±0.505	0.916±0.073
Old Age	1.21±0.122	1.10±0.121	1.23 ±0.244

[a]Human keratinocytes were cultured on type I collagen surface and exposed to hypoxia (H) or normoxia (N) for 20 h. Culture supernatants were collected for zymography analysis of MMP or immunoblotting with respective antibodies. Values are mean±SEM of more than three independent experiments. The p value for MMP-1 induction between young and old age, middle and old age is 0.045 and 0.014, respectively. The p value for MMP-9 induction between young and old age is 0.05. There is no statistic significant induction of MMP-2 among any of the three age groups.

Table II. Hypoxia induced TGF-β receptor expression in keratinocytes isolated from donors of increasing age[a]

	Nomoxia (N)	Hypoxia (H)	Ratio (H/N)
TGFβRI			
Young	3.367±0.323	10.61±1.38	3.16
Middle Age	6.47 ±0.395	11.36±1.48	1.75
Old Age	8.059±1.28	10.5 ±1.71	1.31
TGFβRII			
Young	5.924±0.33	7.206±1.41	1.22
Middle Age	8.621±0.907	10.27 ±1.65	1.19
Old Age	7.892±1.93	7.948±1.36	1.01

[a]Human keratinocytes were cultured on type I collagen surface and exposed to hypoxia (H) or normoxia (N) for 20 h. Cell lysates were analyzed for protein level of TGF-β receptors type I and II by immunoblotting with respective antibodies. Values are mean±SEM of three independent experiments. The p value for TGFβRI induction between young and old age is <0.05. There is no significant hypoxia induced TGFβRII expression among any of the three age groups.

the extracellular domain of TGF-β type II receptor and is capable of binding TGF-β1, 3, and 5 with high affinity and TGF-β2 with moderate-affinity (R&D System). When this peptide was added to the culture medium, hypoxia induction of both MMP-1 and -9 was suppressed (**Fig 6*A*, *B***). TGF-β neutralizing antibody (Genzyme) was also used in a parallel experiment, which gave consistent results as the neutralizing polypeptide (data not shown).

TGF-β function in hypoxia-modulated keratinocyte migration TGF-β has also been suggested to stimulate keratinocyte migration towards provisional wound matrices, by upregulating the expression of the fibronectin receptor α5β1, the vitronectin receptor αvβ5, and the collagen receptor α2β1 (Zambruno *et al*, 1995). We have examined the role of endogenous TGF-β on keratinocyte migration. Keratinocytes were cultured in the presence of TGF-β neutralizing polypeptide, and cell migration was analyzed under both hypoxic and normoxic conditions. The results showed that the hypoxia-stimulated keratinocyte migration of young cells was repressed by neutralizing TGF-β polypeptide (**Fig 7**).

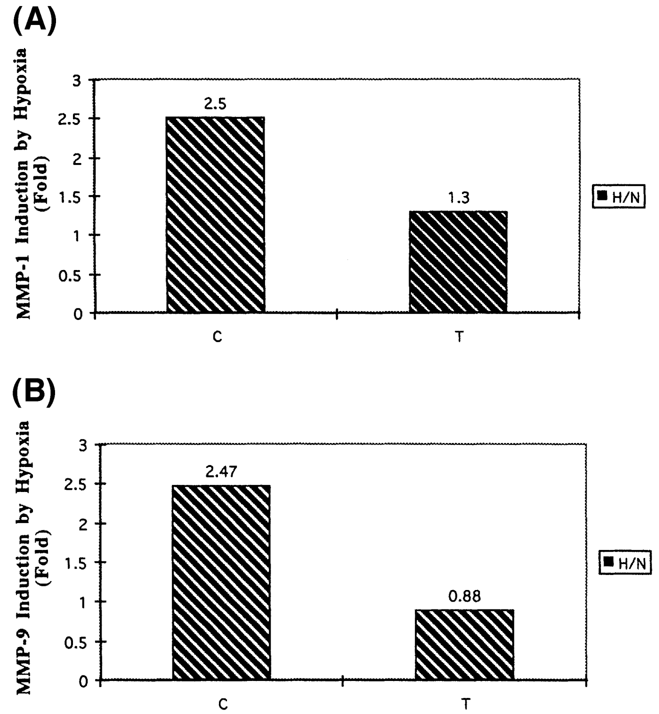

Figure 6. TGF-β neutralizing reagent blocks hypoxia-induced expression of MMP-1 and MMP-9. Keratinocytes isolated from young donors were incubated in the presence (T) or absence (C) of TGF-β neutralizing polypeptide (15 ng per ml) under normoxic (N) or hypoxic (H) for 20 h. Conditioned culture supernatants were collected and examined for MMP-1 (*A*), and MMP-9 (*B*) expression by immunoblotting with respective antibodies. The values for MMP bands were quantitated by densitometry and the relative ratio of expression under hypoxia *vs* normoxia (H/N) was calculated. Average results from three independent experiments are shown.

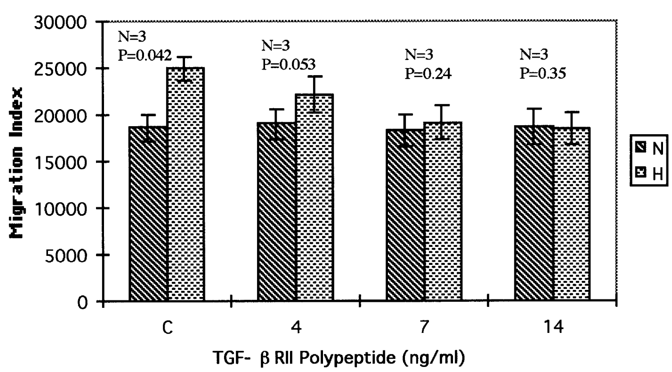

Figure 7. TGF-β neutralizing polypeptide suppresses hypoxia-induced keratinocyte migration. Increasing doses of TGF-β RII polypeptide were incubated with keratinocytes isolated from donors of young age. Keratinocytes were attached to surface of type I collagen (15 μg per ml) coated with gold salt particles. Cells were cultured under hypoxia or normoxia for 20 h prior to fixation with formaldehyde. Migration index was determined by measuring migration track of single cells. Values are mean ± SED of 10 migration tracks from three different donors. C, control, culture medium in the absence of TGF-β RII polypeptide.

DISCUSSION

Chronic wounds are a severe health problem in the elderly population, yet the pathobiology of chronic wounds, especially the key pathogenic factors have not yet been identified. This study presents novel observations with respect to hypoxia-induced differential regulation of keratinocyte migration and MMP/TIMP expression in keratinocytes isolated from young and aged donors. First, we demonstrated that hypoxia significantly reduced the motility of keratinocytes from aged donors. In contrast, cells from young donors manifested an increase in motility under the same hypoxic condition. Second, we analyzed the expression of MMP to identify molecular events associated with the hypoxia-modulated migration. We found that collagenase MMP-1, which is required for keratinocyte migration on type I collagen, was upregulated by hypoxia in young keratinocytes, whereas the level remained unchanged in aged cells. MMP-9, another metalloproteinase that has been implicated in keratinocyte migration and granulation tissue remodeling (Salo *et al*, 1994), was stimulated by hypoxia in young keratinocytes, but the level decreased with hypoxia in aged cells. MMP-2 expression did not respond to hypoxia in young cells cultured on the collagen surface, and there was also no stimulation when cells were cultured on the laminin-5 matrix, a specific substrate for MMP-2 enzymatic activity (Giannelli *et al*, 1997). The MMP-2 expression did increase, however, with increasing chronologic age of donors.

Adequate wound healing requires a careful balance between synthesis and degradation of extracellular matrix proteins (Vaalamo *et al*, 1996; Madlener *et al*, 1998). TIMP, as natural inhibitors of MMP, are expected to play an important part in the counterbalance of MMP activity during wound healing. Indeed, our study showed that young keratinocytes, however, not aged keratinocytes, secreted higher levels of TIMP-1 in response to hypoxia, along with elevated levels of MMP-1 and -9. In contrast, TIMP-2 was not upregulated by hypoxia in either young or aged keratinocytes. It is intriguing that the TIMP-1 level rose along with its targeting MMPs, MMP-1 and -9, molecules that had become inactivated by forming complexes with TIMP-1. Under the same conditions, TIMP-2 did not become upregulated by hypoxia, nor did its targeting enzyme MMP-2. Therefore, it seems that young cells possess a coordinated regulatory mechanism for both MMP and their physiologic inhibitors in the process of tissue repair. This mechanism in young cells allows activation of matrix degradation upon tissue injury. Meanwhile, it activates a counter-control activity to prevent tissue from excessive degradation. Importantly, this regulatory mechanism appears to be lost in aged keratinocytes. Additionally, aged keratinocytes have a higher basal level of MMP-9 and -2 expression than young keratinocytes. Our *in vitro* observations are consistent with previous clinical studies on chronic wounds, in which higher MMP and lower TIMP expression are observed in wound tissue of aged donors compared with the young (Ashcroft *et al*, 1997b,c; Wysocki *et al*, 1993; Bullen *et al*, 1995; Vaalamo *et al*, 1996; Weckroth *et al*, 1996). All together, these data support our hypothesis that tissue hypoxia and age play crucial parts in impaired wound healing.

We further tested the potential involvement of TGF-β signaling components in hypoxia-modulated responses. We found that hypoxia upregulated TGF-β type I receptor expression in young cells, whereas no induction was observed in aged cells. TGF-β type II receptor, on the other hand, did not show any significant upregulation in either young or aged cells. Our results also showed that induction of both MMP-1 and -9 was blocked when cells were coincubated with TGF-β neutralizing reagents. In a functional study, the neutralizing polypeptide was found to suppress hypoxia-induced keratinocyte migration. It was noted that the inhibitory dosage of TGF-β neutralizing reagent to MMP and cell migration was in the same range. These results suggest a promigratory activity of TGF-β in keratinocytes. In cell culture, TGF-β has previously been shown to inhibit keratinocyte proliferation during re-epithelialization, without a significant delay in re-epithelialization (Garlick and Taichman, 1994). Our data may account for, at least partially, the apparent paradox of a TGF-β1-dependent stimulation of epidermal wound healing associated with a growth inhibitory effect on epithelial cells; however, *in vivo* wound healing in aged systems is confounded by other factors. As suggested by our wound healing studies in animal models (Wu *et al*, 1999), ischemic wounds in aged animals did not respond to TGF-β1 treatment. In contrast, the growth factor was able to reverse the wound healing deficit in young animals or in nonischemic wounds. This suggested to us a deficiency in TGF-β signaling in aged animal wounds. The studies reported here, suggest that lack of induction of TGF-β type I receptor by hypoxia in aged keratinocytes, as opposed to upregulation of the same receptor in young cells may account for, at least partially, the differential response to TGF-β treatment in young *vs* aged animals. Therefore, activation or overexpression of TGF-β type I receptor can be a potential therapeutic target.

Although extracellular proteolysis is a prerequisite for normal wound healing, uncontrolled proteolytic tissue destruction appears to be a pathogenic factor in nonhealing wounds (Saarialho, 1998). In fact, extensive extracellular matrix degradation could impair wound closure, as cell–matrix interaction is essential for migration and proliferation. Intact tissue from young individuals has a basal level of MMP expression that becomes upregulated in a timely fashion after injury to facilitate wound repair. Tissue from aged donors has a higher basal level of MMP expression compared with that of the young donors. Meanwhile, there is loss of upregulation in tissue from aged donors during ischemic injury. Therefore, healing with aging is impaired at least 3-fold: (i) an abnormally high basal level of MMP-2 and -9 predisposes the tissue to breakdown; (ii) a lack of response of MMP expression to hypoxia injury impedes the repair processes; and (iii) an discoordinated regulation of TIMP upon injury leads to excessive tissue degradation.

The effect of hypoxia has been previously studied in a number of other systems, including dermal fibroblasts (Falanga *et al*, 1991) and arterial remodeling (Thakker-Varia *et al*, 1998). The novel observation we made in this study relates to the impact of aging on the response to hypoxia. This age-modulated response to the stress of hypoxia leads to differential expression of MMP and TIMP, which *in vivo* would be a partial explanation to the impaired wound healing seen in the elderly with chronic wounds. The underlying altered cellular responses in aging, and their molecular basis has been the subject for intensive research. Recent studies suggest that some of the strongest candidates to explain some of the alterations in aging are the genes that regulate the processes of somatic maintenance and repair, such as the stress–response system (Lithgow and Kirkwood, 1996). An altered stress–response system is consistent with and supported by our findings on the suppression of MMP expression in aged keratinocytes in response to the stress of hypoxia, with a reduction in keratinocyte motility. Our results also provide insights into the clinical observation of the increased incidence of chronic wounds with aging. It seems that the aged system has a diminished capacity to tolerate hypoxia stress, which leads to reduced biochemical responses for wound healing and subsequently a reduced capacity of cells to adapt to and repopulate the wound site. The alteration in response to hypoxia by aged cells has implications beyond chronic wounds to the conditions of myocardial infarction and stroke, in which the magnitude of the injury is related to the ability of the involved tissue to tolerate hypoxia, and ischemia reperfusion injury.

We would like to thank Dr Yong-Jiang Hei for helpful discussions and for his contribution on TIMP-1 and TIMP-2 expression. Our work has been supported by grant R01GM41303 from the National Institute of Health.

REFERENCES

Agren MS: Gelatinase activity during wound healing. *Br J Dermatol* 131:634–640, 1994

Arthur WT, Vernon RB, Sage EH, Reed MJ: Growth factors reverse the impaired sprouting of microvessels from aged mice. *Microvasc Res* 55:260–270, 1998

Ashcroft GS, Horan MA, Ferguson MW: Aging is associated with reduced deposition of specific extracellular matrix components, an upregulation of angiogenesis, and an altered inflammatory response in a murine incisional wound healing model. *J Invest Dermatol* 108:430–437, 1997a

Ashcroft GS, Herrick SE, Tarnuzzer RW, Horan MA, Schultz GS, Ferguson MW: Human ageing impairs injury-induced in vivo expression of tissue inhibitor of matrix metalloproteinases (TIMP) –1 and –2 proteins and mRNA. *J Pathol* 183:169–176, 1997b

Ashcroft GS, Horan MA, Herrick SE, Tarnuzzer RW, Schultz GS, Ferguson MWJ: Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMP) in normal skin and acute cutaneous wounds of healthy humans. *Cell Tissue Res* 290:581–591, 1997c

Bullen EC, Longaker MT, Updike DL, Benton R, Ladin D, Hou Z, Howard EW: Tissue inhibitor of metalloproteinases-1 is decreased and activated gelatinases are increased in chronic wounds. *J Invest Dermatol* 104:236–240, 1995

Carter WG, Ryan MC, Gahr PJ: Epiligrin, a new cell adhesion ligand for integrin alpha 3 beta 1 in epithelial basement membranes. *Cell* 65:599–610, 1991

Cavani A, Zambruno G, Marconi A, Manca V, Marchetti M, Giannetti A: Distinctive integrin expression in the newly forming epidermis during wound healing in humans. *Invest Dermatol* 101:600–604, 1993

Falanga V, Qian SW, Darnelpour D, Katz MH, Roberts AB, Sporn MB: Hyporra upregulates the synthesis of TGF-beta by human dermal fibroblasts. *J Invest Dermatol* 97:634–637, 1991

Franklin B, Poyton RO: Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* 76:839–885, 1996

Frantz RA, Gardner S: Elderly skin care: principles of chronic wound care. *J Gerontol Nurs* 20:35–44, 1994

Garlick JA, Taichman LB: Effect of TGF-β 1 on re-epithelialization of human keratinocytes in vitro: an organotypic model. *J Invest Dermatol* 103:554–559, 1994

Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V: Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* 277:225–228, 1997

Grinnell F: Wound repair, keratinocyte activation and integrin modulation. *J Cell Sci* 101:1–5, 1992

Howard EW, Banda MJ: Binding of tissue inhibitor of metalloproteinases 2 to two distinct sites on human 72-kDa gelatinase. Identification of a stabilization site. *J Biol Chem* 266:17972–17977, 1991

Lithgow GJ, Kirkwood TB: Mechanisms and evolution of aging. *Science* 273:80, 1996

Liu X, Wu H, Byrne M, Jeffrey J, Krane S, Jaenisch R: A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. *J Cell Biol* 130:227–237, 1995

Madlener M, Parks WC, Werner S: Matrix metalloproteinases (MMP) and their physiological inhibitors (TIMP) are differentially expressed during excisional skin wound repair. *Exp Cell Res* 242:201–210, 1998

Mauviel A, Chung KY, Agarwal A, Tamai K, Uitto J: Cell-specific induction of distinct oncogenes of the Jun family is responsible for differential regulation of collagenase gene expression by transforming growth factor-β in fibroblasts and keratinocytes. *J Biol Chem* 271:10917–10923, 1996

Moses MA, Marikovsky M, Harper JW, Vogt P, Eriksson E, Klagsbrun M, Langer R: Temporal study of the activity of matrix metalloproteinases and their endogenous inhibitors during wound healing. *J Cell Biochem* 60:379–386, 1996

Murphy G, Reynolds JJ. Extracellular matrix degradation. In: Royce PM, Steinmann B (eds). *Connective Tissue and its Heritable Disorders*. New York: Wiley, 1993, pp 287–316

Niinikoski J, Hunt TK, Dunphy JE: Oxygen supply in healing tissue. *Am J Surg* 123:247–252, 1972

O'Toole EA, Marinkovich MP, Peavey CL, Amieva MR, Furthmayr H, Mustoe TA, Woodley DT: Hypoxia increases human keratinocyte motility on connective tissue. *J Clin Invest* 100:2881–2891, 1997

Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG, Parks WC: The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J Cell Biol* 137:1445–1457, 1997

Rougier JP, Moullier P, Piedagnel R, Ronco PM: Hyperosmolality suppresses but TGF beta 1 increases MMP9 in human peritoneal mesothelial cells. *Kidney Int* 51:337–347, 1997

Saarialho-Kere UK: Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. *Arch Dermatol Res* 290(Suppl.):S47–S54, 1998

Salo T, Lyons JG, Rahemtulla F, Birkedal-Hansen H, Larjava H: Transforming growth factor-β 1 up-regulates type IV collagenase expression in cultured human keratinocytes. *J Biol Chem* 266:11436–11441, 1991

Salo T, Makela M, Kylmaniemi M, Autio-Harmainen H, Larjava H: Expression of matrix metalloproteinase-2 and –9 during early human wound healing. *Lab Invest* 70:176–182, 1994

Sudbeck BD, Pilcher BK, Welgus HG, Parks WC: Induction and repression of collagenase-1 by keratinocytes is controlled by distinct components of different extracellular matrix compartments. *J Biol Chem* 272:22103–22110, 1997

Thakker-Varia S, Tozzi CA, Poiani GJ, Babiarz JP, Tatem L, Wilson FJ, Riley DJ: Expression of matrix-degrading enzymes in pulmonary vascular remodeling in the rat. *Am J Physiol* 275(2 Part 1):L398–L406, 1998

Uria JA, Jimenez MG, Balbin M, Freije JMP, Lopez-Otin C: Differential effects of transforming growth factor-beta on the expression of collagenase-1 and collagenase-3 in human fibroblasts. *J Biol Chem* 273:9769–9777, 1998

Vaalamo M, Weckroth M, Puolakkainen P, Kere J, Saarinen P, Lauharanta J, Saarialho-Kere UK: Patterns of matrix metalloproteinase and TIMP-1 expression in chronic and normally healing human cutaneous wounds. *Br J Dermatol* 135:52–59, 1996

Vaalamo M, Mattila L, Johansson N, Kariniemi AL, Karjalainen-Lindsberg ML, Kahari VM, Saarialho-Kere U: Distinct populations of stromal cells express collagenase-3 (MMP-13) and collagenase-1 (MMP-1) in chronic ulcers but not in normally healing wounds. *J Invest Dermatol* 109:96–101, 1997

Van de Kerkhof PC, Bergen B, Spruijt K, Kuiper JP: Age-related changes in wound healing. *Clin Exp Dermatol* 19:369–374, 1994

Weckroth M, Vaheri A, Lauharanta J, Sorsa T, Konttinen YT: Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. *J Invest Dermatol* 106:1119–1124, 1996

Welgus HG, Jeffery JJ, Eisen AZ: The collagen substrate specificity of human skin fibroblast collagenase. *J Biol Chem* 256:9511–9515, 1981

Woessner JF Jr: Matrix metalloproteinases and their inhibitors in connective tissue remodelling. *FASEB J* 5:2145–2154, 1991

Woodley DT, Bachmann PM, O'Keefe EJ: Laminin inhibits human keratinocyte migration. *J Cell Physiol* 136:140–146, 1988

Wu H, Byrne MH, Stacey A, Goldring MB, Birkhead JR, Jaenisch R, Krane SM: Generation of collagenase-resistant collagen by site-directed mutagenesis of murine pro alpha 1 (I) collagen gene. *Proc Natl Acad Sci USA* 87:5888–5892, 1990

Wu L, Xia Y-P, Siddiqui A, Roth S, Gruskin E, Mustoe T: TGF-beta 1 fails to stimulate wound healing and impairs its signal transduction in an aged ischemic ulcer model: the importance of oxygen and age. *Am J Pathol* 154:301–309, 1999

Wysocki AB, Staiano-Coico L, Grinnell F: Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol* 101:64–68, 1993

Zambruno G, Marchisio PC, Marconi A, Vaschieri C, Melchiori A, De Giannetti A, Luca M: Transforming growth factor-β 1 modulates beta 1 and beta 5 integrin receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: implications for wound healing. *J Cell Biol* 129:853–865, 1995